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Short Communication

The Suitability of a Quantitative Spectrophotometric Assay for Phenylalanine Ammonia-lyase Activity in Barley, Buckwheat, and Pea Seedlings[1]

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ABSTRACT

It has been suggested by others that the spectrophotometric assay of phenylalanine ammonia-lyase based on changes in absorbance at 290 nm may be complicated by a side reaction involving transamination from phenylalanine onto α-keto acids. This would lead to the production of phenylpyruvate which would spontaneously tautomerize and form an enol borate complex absorbing at this wavelength. We find that the inclusion of 1 ml of either 60 μM α-ketoglutarate or 500 μM phenylpyruvate in our 3-ml reaction mixtures has no significant effect on the spectrophotometric assay of phenylalanine ammonia-lyase in shoots from young seedlings of barley (*Hordeum vulgare*), buckwheat (*Fagopyrum esculentum*), or pea (*Pisum sativum*). Although these side reactions may be involved in preparations with very low enzyme activity, the spectrophotometric determination of phenylalanine ammonia-lyase based on changes in absorbance at 290 nm appears to be a reliable and sensitive technique in these seedlings.

Erez (3) has pointed to possible errors in the widely used spectrophotometric assay of phenylalanine ammonia-lyase based on increased absorbance at 290 nm of cell-free preparations in pH 8.8 borate buffer (9). PAL[2] deaminates L-phenylalanine to produce *trans*-cinnamic acid which absorbs strongly at 290 nm under these conditions. He suggests that under optimal conditions for PAL activity and in the presence of aminotransferases and α-keto acids, phenylalanine may be diverted into phenylpyruvate which will tautomerize and form an enol tautomer-borate complex interfering with the assay. As we have used this spectrophotometric assay for the quantitative determination of PAL in barley (6–8), we attempted to determine the extent to which traces of α-keto acids present in our crude extracts might lead to the formation of complexes interfering in the assay. PAL levels in peas and in buckwheat have been examined by several techniques (1, 2), and these plants were included for comparative purposes.

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[2] Abbreviation: PAL: phenylalanine ammonia-lyase.

MATERIALS AND METHODS

Atlas 68 barley (*Hordeum vulgare*, a gift of Dr. Burt Ray, University of California at Davis), Penquad buckwheat (*Fagopyrum esculentum*, a gift of Dr. Harold Marshall of Pennsylvania State University), and Alaska peas (*Pisum sativum* from General Biological Inc., Chicago, Ill.) were grown in the dark on water-saturated vermiculite in 23 C incubators under high humidity conditions (8). All manipulations of the etiolated plants, from soaking the seeds through centrifuging the enzyme preparations, were carried out in the dark or under a dim green safelight (8).

Whole shoots were assayed by a technique modified from Zucker (9). Four shoots were excised at the point of emergence from the seed or caryopsis, weighed, and thoroughly ground in a chilled Ten Broeck homogenizer containing 8 ml of cold 25 mM borate buffer (pH 8.8), 23 μl of mercaptoethanol, and 0.3 g of Polyclar AT which had been soaked overnight in mercaptoethanol-containing buffer before use. The homogenates were centrifuged at 4 C for 30 min at 30,900*g*, and the supernatant was used as a source of enzyme. In the α-ketoglutarate experiments, the supernatants from three preparations were pooled to provide sufficient uniform material for replicate determinations.

The PAL assay system routinely consisted of 1 ml of the supernatant, 1 ml of 50 mM L-phenylalanine, and 1 ml of buffer. Controls contained buffer in place of phenylalanine. The change in absorbance at 290 nm was monitored in 1-cm light path cells at 10- to 15-min intervals, following Zucker's technique (9), for 1 hr or more at 40 C. Under these conditions, a change in absorbance of 0.01 was found to be equivalent to the production of 3.09 nmoles of cinnamic acid.

To test the interference at 290 nm attributable to the formation of phenylpyruvate complexes, 1 ml of the buffer was replaced with either 1 ml of 60 μM α-ketoglutarate dissolved in borate buffer, or 1 ml of freshly prepared 500 μM enol tautomer of phenylpyruvate (4) dissolved in water and adjusted to pH 8.8 with NaOH. All analytical reagents were obtained from Sigma Chemical Co.

Each data point is the mean of at least five determinations, each done in triplicate. PAL activity for barley in the assays containing phenylpyruvate is corrected for the negative correlation coefficient between fresh weight and PAL content per gram. For example, dark-grown four-plumule barley samples weighing 0.35 g have approximately 17% less PAL/g fresh weight than do four-plumule samples weighing 0.30 g (6, 7).

RESULTS AND DISCUSSION

In spectrophotometric assays of PAL from peach apices (3) absorbance at 290 nm increased more rapidly in those containing both α-ketoglutarate and phenylalanine than in those containing only phenylalanine. These preparations also form about 0.029 nmole of glutamate/min·ml when both α-ketoglutarate and phenylalanine are added (3). As an equivalent amount of phenylpyruvate would be formed in this reaction, this would lead to an increased absorbance of about 0.00003 per min using ϵ 290 nm of 2950 for phenylpyruvate in the pH 8.8 borate buffer (3). This is considerably less than the error term in spectrophotometric assays for PAL from most seedlings (2, 6–9). This level of phenylpyruvate formation is similar to that found in crude extracts of pea shoots (5).

We do not detect significant differences attributable to the inclusion of α-ketoglutarate in our PAL assay systems from barley, buckwheat, or pea (Table I). The levels of phenylpyruvate formed in crude preparations of pea shoots are so low (5) and the levels of PAL in these preparations are, by comparison, so high when measured by either spectrophotometric or labeling (1) techniques, that any interference due to phenylpyruvate complexes would be insignificant. The PAL values that we find for barley, buckwheat, and peas seem to be quite representative for a broad range of seedlings (1, 2). It should be noted that the addition of α-ketoglutarate to borate-containing reaction mixtures may under certain conditions give rise to increased absorbance at 290 nm not attributable to the formation of phenylpyruvate complexes (4).

To test the interaction of phenylpyruvate with the enzyme system, we added 1 ml of 500 μM phenylpyruvate to an enzyme preparation from barley. As expected, the added phenylpyruvate immediately increased the absorbance at 290 nm about 0.12 absorbance units and this absorbance remained unchanged throughout a 45-min period. Another aliquot of the enzyme was provided phenylalanine at zero time. At 45 min 1 ml of buffer was added to the phenylalanine-containing reaction and 1 ml of phenylalanine added to the phenylpyruvate-containing reaction. Changes in absorbance at 290 nm were then followed between 45 and 105 min. The phenylalanine plus buffer and phenylpyruvate plus phenylalanine samples increased in absorbance at 290 nm at rates corresponding to 95 and 87 nmoles cinnamic acid/min·g fresh weight, respectively. These rates are lower than those reported for barley in Table I, as PAL activity declines somewhat after such long periods of assay. The slight inhibitory effect of adding phenylpyruvate to the reaction mixtures was not statistically significant. Thus neither α-ketoglutarate nor phenylpyruvate at these levels (Table I; ref. 3) significantly interfere with the spectrophotometric assay of PAL.

The peach apex preparations have extremely low PAL activity, producing approximately 0.16 nmole of cinnamic acid/min in the reaction mixture (3). Barley, buckwheat, and pea seedlings have much higher levels of PAL producing about 111.1, 7.4, and 5.5 nmoles, respectively, of cinnamic acid/min. PAL activity of these seedlings determined by spectral techniques (Table I) are in good agreement with levels obtained using ^{14}C labeling techniques (1).

For all of the above reasons, although it may apply in certain cases of extremely low PAL activity, we do not consider that the enzymatic production of phenylpyruvate and the subsequent formation of complexes absorbing at 290 nm are significantly involved in the spectrophotometric determination of PAL in crude extracts prepared from shoots of barley, buckwheat, or pea seedlings.

Table I. *Effect of Adding α-Ketoglutarate to PAL System from Dark-grown Shoots of Barley, Pea, or Buckwheat*

Four 6-day-old shoots were weighed, homogenized in 8 ml of cold pH 8.8 borate buffer containing mercaptoethanol and Polyclar AT, and the filtrate was centrifuged at 30,900*g* for 30 min at 4 C. Reaction mixtures contained 1 ml of supernatant, 1 ml of 50 mM L-phenylalanine (Phe) and/or 60 μM α-ketoglutarate (α-KG) and, if necessary, buffer to a total volume of 3 ml. Absorbance at 290 nm was monitored for 60 min at 40 C against a blank containing enzyme and buffer. A change in absorbance of 0.01 under these conditions is equivalent to the production of 3.09 nmoles of cinnamic acid.

Plant	Substrate	Rate of Cinnamic Acid Formation Based on Changes in Absorbance at 290 nm	Standard Deviation[1]
		nmoles/min·g fresh wt	
Barley	Phe	111.1	10.6
	Phe + α-KG	105.7	9.3
	α-KG	0.0	
Buckwheat	Phe	7.4	1.7
	Phe + α-KG	6.7	1.3
	α-KG	0.0	
Pea	Phe	5.5	1.3
	Phe + α-KG	5.1	1.2
	α-KG	0.0	

[1] In no cases were there any significant differences at the 5% level attributable to the addition of α-KG to the Phe containing reaction mixtures.

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